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Antidiabetic activities of menthone on insulin resistant in 3T3-L1 adipocytes

M. Revathi
Research scholar, Department of Biochemistry, Sri Akilandeswari Womens College, Vandavasi-604 408

K. Padmalochana
Department of Biochemistry, Sri AkilandeswariWomens College,Vandavasi--604 408
Corresponding author email: devnathyazhini@gmail.com

C. Sankaranarayanan
Department of Biochemistry & Biotechnology, Annamalai University, Annamalai Nagar, Chidambaram- 608002

S. Nalini
Department of Microbiology, Shree Ragavendra Arts and Science College, Keezhamoongiladi, Chidambaram-608102

P. Ramya
Department of Microbiology, Shree Ragavendra Arts and Science College, Keezhamoongiladi, Chidambaram-608102

***Abstract*---**Background: Diabetes mellitus (DM) is a metabolic disorder characterised by chronic hyperglycaemia and metabolic consequences caused by insulin defects. Adipose tissue is an endocrine organ that influences glucose and lipid metabolism by releasing adipokines; adipose tissue is vital for the life of mammals. It represents the major source of fatty acid. Brown adipose tissue (BAT) and white adipose tissue (WAT) are the basic adipose tissue forms. They have different functions and cellular compositions and localization. They are essentially adversarial in terms of functionality. WAT is known to contain the most body fat; its involvement in the storage of excess dietary triglycerides. It is the source of free fatty aid used as energy substrates for the generation through oxidative phosphate of (ATP) high-energy bonds. During insulin resistance, the secretion of the hormone from the pancreatic islet cells cannot trigger glucose uptake in metabolic tissues, leading to elevated blood glucose and insulin levels. Menthone is cyclic monoterpenes and is reported to have several biological activities. This study, explores the potential

antidiabetic effects invitro of menthone in the 3T3L1 adipocytes. Methods: The differentiation of 3T3-L1 preadipocytes into mature adipocytes were cultured and maintained. To investigate their half maximal inhibitory concentration (IC_{50}) value using thiazoyl blue tetrazolium bromide (MTT assay). Also the effect of methone on lipid accumulation in mature 3T3-L1 preadipocytes were evaluated by oil Red O staining assay. In addition, effect of menthone on antioxidative parameters were studied by Mitochondrial membrane potential (MMP) assay, Reactive oxygen species (ROS), catalase enzymatic activity, and Glutathione peroxidase (GPx) assay in 3T3L-1cell line. Results:The results revealed the treatment (25 mM/L glucose + 0.6 nm/L insulin + 63.22 µM/ml of menthone) with menthone resulted in a significantly increased lipid accumulation in the cells. The IC_{50} value for menthone was observed as 70.26 µM/ml. Menthone treatment has significantly reduced ROS production and increased catalase, GPx activity. Conclusion: In conclusion, these results suggest the effect of menthone in the insulin signalling pathway and enhance the antioxidant levels in 3T3L-1 adipocytic cells.

***Keywords*---**Antidiabetic, insulin resistant, 3T3-L1 adipocytes.

Introduction

Diabetes is a metaboilic disorder with chronic hyperglyceamia featured by metabolic outcomes owing to insufficient insulin secretion or insulin defect. Chronic hyperglycemia causes cardiovascular disease in diabetes mellitus. Diabetes mellitus is one of the most frequent and fastest growing diseases globally with 693 million adults expected to be affected by 2045 (Cho et al., 2018). The prevalence of diabetic mellitus was 303, with rural residents having 2.0 percent and urban residents having 4.6 percent. The mean blood glucose level and diabetes mellitus were substantially higher for urban residents than rural residents (Animaw and Seyoum, 2017). Retinopathy, neuropathy, and nephropathy are all complications associated with diabetes (Nathan, 1993). Adipose tissue secretes bioactive peptides named adipokinase. Increased adipokinase production affects various activities, including hunger and energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism, and haemostasis (Balistreri et al., 2010).

Adipose tissue is an endocrine organ that influences both glucose and lipid metabolism (Kershaw et al., 2004; Scherer, 2006) by releasing adipokines. Insulin acts on adipose tissue by stimulating glucose uptake and triglyceride synthesis and by suppressing triglyceride hydrolysis and release of FFA and glycerol into the circulation (Saponaro et al., 2015; Boden, 2008). Adipose tissue insulin resistance (Adipo-IR), that is, the impaired suppression of lipolysis in the presence of high insulin levels, has been associated with glucose intolerance, and elevated plasma FFA levels have been shown to impair muscle insulin signaling, promote hepatic gluconeogenesis, and impair glucose-stimulated insulin response (Kashyap et al., 2003; Ferrannini et al., 1983).

Adipocyte function in diabetes is extensively studied using the 3T3-L1 adipocyte model. Cell lines as in vitro model systems have been provided opportunity to understand effects of plant extracts on animal and human health for a long time. Recently, Lavanya et al., (2022) reported the *invitro* activity on glucose uptake of 3T3L-1 adipocytes mediated via PPARγ through *invitro* and molecular docking studies.

The Mentha(mint) are of economically important members of *Lamiaceae* family and have a long history of usageas a medicinal ingredient. Menthone, is a cyclic monoterpene found in the Mentha genus (Kamatou et al., 2013). Menthone is the major essential oil that has been proven to have several biological activities such as antimicrobial, antifungal, anticancer and inflammatory effects (Kamatou et al., 2013). The cooling minty flavour and smell of plants are due to menthone(Lawrence, 2013). The biological activities of the menthone, on the other hand, are still barely understood. Hence, we aimed to investigate the antidiabetic effect of menthone on 3T3-L1 adipocytes.

Materials and Methods

Chemical

The Gibco Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and antibiotic solution were from Gibco (USA), Menthone, DCFH-DA, JC-10 and 1x PBS was procured from Himedia, India. Tris-HCL, EDTA, Glucose, Sodium Chloride, H_2O_2 were Purchased from Merk, USA. GSH- Glutathione GSSG-glutathione oxidised form, β-NADPH- β- Nicotinamide Adenine Dinucleotide Phosphate, EDTA, SodiumeAzide H_2O_2 Were Purchased from SRL India. Oil red O powder was obtained from Sigma-Aldrich,USA.

Cell culture and differentiation of 3T3-L1 into mature adipocytes

3T3 L1 adipocytes were purchased from NCCS, Pune.The cells were cultured in liquid medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycinand maintained under an atmosphere of 5% CO_2 at 37 ^oC.The differentiation of 3T3-L1 preadipocytes into mature adipocytes was cultured and maintained (Green and Kehinde, 1975). Differentiation medium was prepared 90% DMEM supplemented with 10% FBS, 1% L-Glutamine, 1% Penicillin+streptomycin antibiotics-containing DEX (1µM), Insulin (1 µg/mL), and IBMX (0.5 mM). First, 3T3-L1 cells were seeded preadipocyte expansion medium at 8x104 in a 6-well plate containing 1 mL medium until they reached confluence. After 48 h for the induction of differentiation 3T3-L1 preadipocytes were cultured with a differentiation medium. After 72 h of the induction of insulin resistance, the differentiation medium was removed and added by adipocyte maintenance DMEM supplemented with 10% FBS and insulin (1µg/mL) for another 48 h. After 5 days of the induction of differentiation, the fresh adipocyte maintenance medium was replaced every two days until 14 days.

For further experiments the cells were grouped in to four and hyperglycaemic condition was induced by treating cells with 25 mM/L glucose + 0.6 nm/L insulin. Further the groups were followed; Group 1: 3T3-L1 preadipocytes + 5

mM/L glucose + 0.6 nm/L insulin, Group 2: 3T3-L1 mature adipocytes + 25 mM/L glucose + 0.6 nm/L insulin, Group 3: 3T3-L1 mature adipocytes+25 mM/L glucose + 0.6 nm/L insulin + 63.22 µM/ml of Menthone; Group 4: 3T3-L1 mature adipocytes+25 mM/L glucose + 0.6 nm/L insulin + 63.22 µM/ml of Rosiglitazone.

The cytotoxic effect of menthone on 3T3-L1adipocytes

The toxicity of menthone on 3T3-L1 mature adipocytes cells was determined by MTT assay. The 3T3 L1 mature adipocytescells were inoculated (1 x 10^5 cells/well) in a 96-well plate and cultivated for 24 hours in a humified environment. Further, the cells were pre-treated with 25 mM Glucose and administrated with various increasing doses (10-100 µM/ml) of menthone for 24 h at humified incubation. After the incubation period, MTT (20 µL of 5 mg/ml) was added to each well, and the cells were incubated for another 2-4 h until purple precipitates were visible under an inverted microscope. Then the medium was aspirated, and the resulting formazan was diluted by 100 µL of DMSO into all wells for 5 min. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC_{50} value were calculated using GraphPad Prism 6.0 software (USA).

Effect of menthone on lipid accumulation in 3T3-L1 adipocytes

In brief, the 3T3-L1 mature adipocytescells were seeded in 6 wells plate (3 x 10^5) and cultured in humified condition for 24 h. After the cells were uniformly grown, the group 3 cells were administrated with 63.22 µM/ml of menthone, the group 4 cells were administrated with 0.1 µM Rosiglitazone for 24 h in CO_2 condition. The group 1 and group 2 cells remained without treatment. After 24 h of treatment, the cells were washed with PBS and fixed with the 4%c of formalin in PBS 0.05M, and again washed with the 60% isopropanol for 2 min and stained with a filtered 0.35% Oil Red O solution in 60% isopropanol for 10 min at room temperature. Further, the cells were washed with PBS solution and observed under Olympus light microscope.

Measurement of intracellular ROS

The ROS activities induced in 3T3-L1 mature adipocytescells were ascertained by treating the cells with DCFH-DA (Wang et al., 1993). In brief, the cells were seeded in 6 wells plate (3 x 10^4) and cultured in humified condition for 24 h. After the cells were uniformly grown, the group 3 cells were administrated with 63.22 µM/ml of menthone, the group 4 cells were administrated with 0.1 µM Rosiglitazone for 24 h in CO_2 condition. The group 1 and group 2 cells remained without treatment. Afterwards, the cells were stained with 100 µL of DCFH-DA for 10 min at 37 °C under dark conditions, later, the cells were washed with PBS and observed under fluorescence microscopy equipped with a digital camera and appropriate filters.

Measurement of mitochondrial membrane potential

According to the protocol recommended previously, the JC-10 dye was used to stain the cells to explore the MMP (Johnson et al., 1980). In brief, the 3T3-L1

mature adipocytescells were seeded in 6 wells plate and cultured in humified condition for 24 h. After the cells were uniformly grown, the group 3 cells were administrated with 63.22 µM/ml of menthone,and the group 4 cells were administrated with 0.1 µM Rosiglitazone for 24 h in CO_2 condition. The group 1 and group 2 cells remained without treatment. After treatment, the cells were stained with JC-10 for 30 min. Then the cells were observed for the mitochondrial membrane alteration under fluorescence microscopy equipped with a digital camera and appropriate filters.

Estimation of Catalase Activity

The menthone sample was tested for catalase enzymatic activity in 3T3-L1 mature adipocytescells. Briefly, the cells were plated at a density of 1×10^5 cells/ml into the 24-well tissue culture plate in a DMEM medium containing 10 % FBS and 1% antibiotic solution for 24hours at 37°C. The wells were washed with sterile PBS and treated with test samples (Group 1to group 4) in a serum-free DMEM medium. Each sample was replicated three times, and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After treatment the cells were lysed using ice-cold homogenate medium (pH 7.4 contain 0.01 mol/L Tris- HCL, 0.0001 mol/l EDTA-2Na, 0.01mol/L Sucrose, 0.8% Sodium chloride solution) and centrifuged 5000 xg for 10 min at 2-4°C to obtained supernatant. About 75 µl of samples were used for assayCAT enzymatic activity was quantified in a spectrophotometer cuvette. After adding 330 µL H_2O_2 (30 mM) and adjusting to 1 mL with PBS, the H_2O_2 absorbance change was continuously measured at 240 nm every 30 s with a spectrophotometer (Lambda EZ-150; Perkin Elmer Company; Waltham, MA, USA). The results of enzymatic activity were reported as the U min-1/mg protein.

Estimation of GPxactivity

The 3T3 L1cells (20,000–50,000 cells/well) were plated to a 24 well plate and incubated for 24 hr in a DMEM growth medium. After incubation, the plate was washed with PBS and treated (Group 1 to Group 4) in a serum-free DMEM medium. Again, the plate was incubated at 37°C in a humidified 5% CO_2 incubator for 24 hrs.The samples were lysed in a lysis medium. Briefly, cells were lysed with 0.9 ml of ice-cold lysis medium (pH 7.4 contain 0.01 mol/L Tris- HCL, 0.0001 mol/l EDTA-2Na, 0.01mol/L Sucrose, 0.8% Sodium chloride solution) and centrifuged 5000 RPM for 10 min at 2-4^{0}C to obtained supernatant. 50 µl of samples were used for the assay.

Result and Discussion

The Cytotoxic effect of the menthone on3T3L-1 adipocytes

The MTT assay was carried out to evaluate the cytotoxic effects of the menthone on 3T3-L1 mature adipocytes cells. As shown in Fig.1, the concentration-dependent cytotoxicity in 3T3L-1 cells were demonstrated by menthone. Briefly, the cells were treated with different concentrations of menthone for 24h, and the cell viability decreased with an increase in menthone concentration. The 50% inhibition of cell growth (IC_{50}) was calculated as 70.26 µM/ml. The data observed

confirmed the effect of menthone on the viability of 3T3L-1 in a dose-dependent manner.

Effect of Menthone on Lipid accumulation in 3T3L-1 adipocytes

Oil red O staining was performed to evaluate the effect of menthone on the lipid accumulation ability of 3T3-L1 adipocytes. The staining was carried out to distinguish the lipid droplet in adipocytes treated with menthone at various concentrations for 24 h with the oil red O kit. The results showed an increased lipid accumulation in mature adipose cells compared to the preadipose cells. However, the treatment with menthone resulted in a significant increase in lipid accumulation in the cells. Compared to menthone treatment, the Rosiglitazone treatment showed a more effective increase in lipid accumulation in mature adipose cells (Fig. 2). The most common use of quantitative oil red O staining is to determine the ability of cultivated preadipocytes to differentiate under various experimental conditions (Kraus et al., 2016).

Inhibitory effect of Menthone on ROS production

Several metabolic diseases, including diabetes, insulin resistance, cardiovascular disease, and obesity, have been associated with oxidative stress. The ROS production in 3T3L-1 cells was determined by DCFH-DA staining. In contrast to the control cells (group 1), the depth of green fluorescence was significantly increased in group 2 cells. However, fluorescence intensity was significantly reduced when treated with Mentone in insulin-resistant group 3 cells. Despite this, the treatment of Rosiglitazone in insulin-resistant group 4 cells more effectively reduced the ROS production, as evidenced by decreased fluorescence. The observation reveals that a decreased intracellular ROS level was induced by Menthone and Rosiglitazone in Adipocyte cells (Fig. 3).

Effect of Menthone on MMP

The results showed that menthone treatment altered the mitochondrial membrane potential in 3T3L-1 adipocytes(Fig. 4). The menthone treatment significantly changed the MMP in group 3 cells, indicating changes the mitochondrial membrane integrity, evidenced by the reduced fluorescence depth. Moreover, the Rosiglitazone administration significantly decreased the membrane integrity (group 4). The mitochondrial membrane potential is directly proportional to ROS formation: the MMP starting at $\Delta\Psi m>140$ mV, relative oxygen potential increases rapidly (Liu, 1999). Hyperglycemia in diabetic individuals can cause mitochondrial superoxide generation, which contributes to the development of problems and plays a role during normal glycemia periods after hyperglycemia (Brownlee, 2001).Wang et al., (2015) used rhodamine 123 dye to evaluate the MMP in A549 cells and found a dose-dependent increase in green fluorescence. The authors found mitochondrial membrane depolarisation and apoptosis after activating the intrinsic mitochondrial pathway.

Effect of Menthone on Catalaseand GPxactivity

Cellular antioxidant enzymes, including Gpx, and CAT, play an essential role in the defence system against oxidative stress. CAT catalyses the dismutation of two molecules of H_2O_2 into two molecules of water and one molecule of oxygen. Regarding the catalase activity, the menthone caused an augmentation in the enzyme activity compared to the group 2 cells (Fig .5). An increase in GP_X level was observed (0.32±0.02) in menthone administrated group 3, which is less than the level of GPx obtained after treatment with control(0.21 ±0.01).The GP_X level of 0.44±0.02 was observed in Rosiglitazone treated group 4(Fig .6).

Conclusion

The present study reveals the antioxidative effect of menthone on insulin resistantce in 3T3L-1 adipocytes. The menthone significantly reduced the intracellular ROS levels and increased lipid accumulation in insulin resistance in 3T3L-1 adipocytes. In addition, the menthone has significantly reduced the availability of mature 3T3L-1 adipocytes in a concentration dependant manner. Moreover, the treatment of menthone increased mitochondrial membrane potential in 3T3L-1 adipocytes, reduced ROS production, and increased the catalase and GPx enzyme activity. The results show that the menthone effectively reduced insulin resistance by enhancing GPx and catalase activity and ROS reduction in mature 3T3L-1 adipocytes cells.

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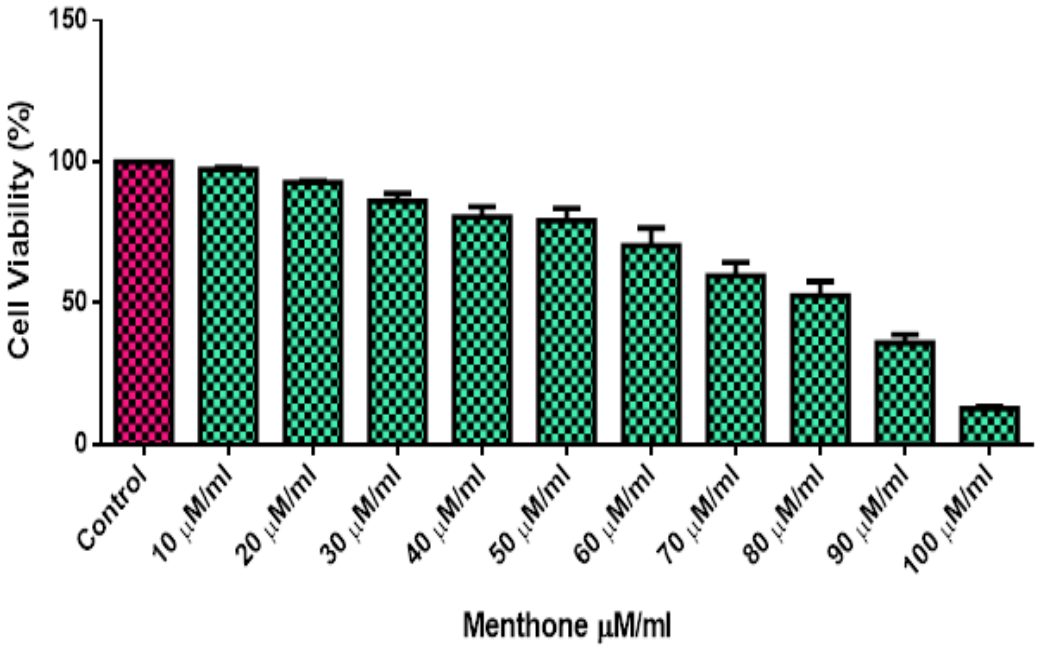


Fig 1. Cytotoxicity of Menthone on 3T3L-1 adipocytic cells. Values were expressed in mean ± standard error.

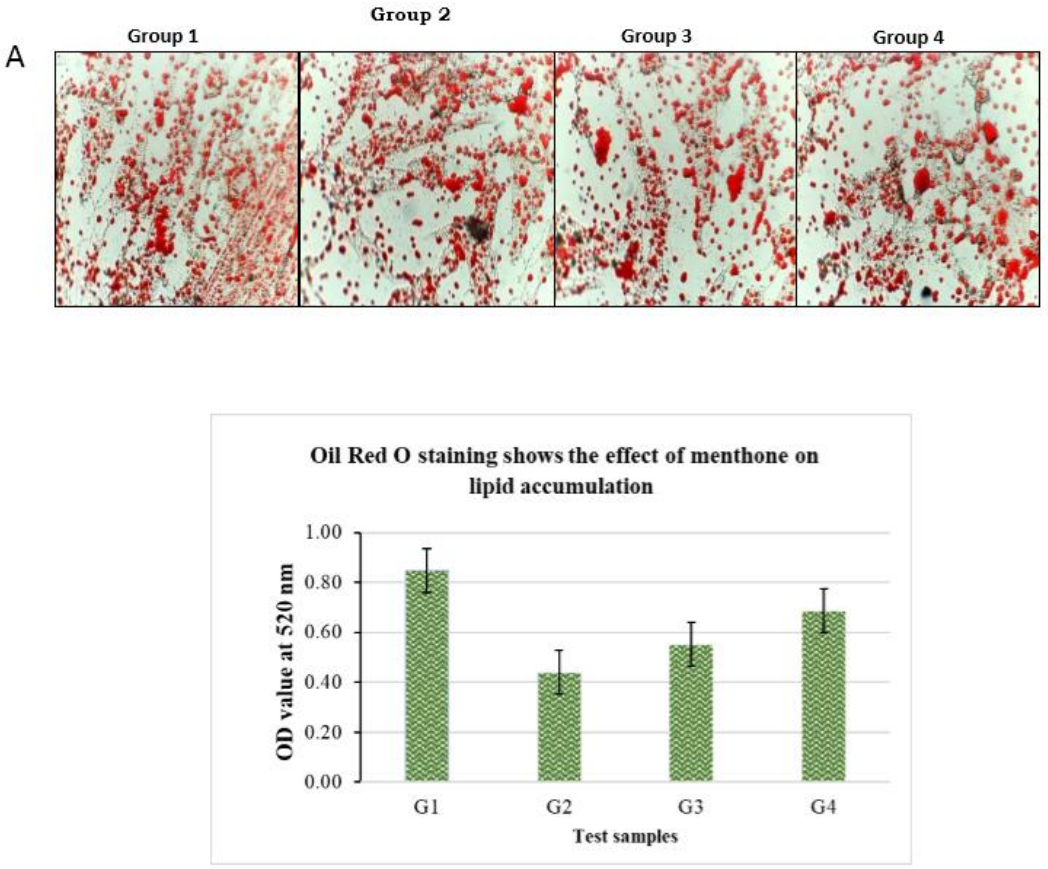
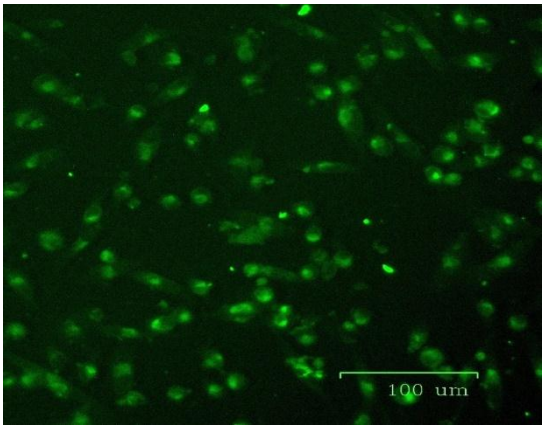
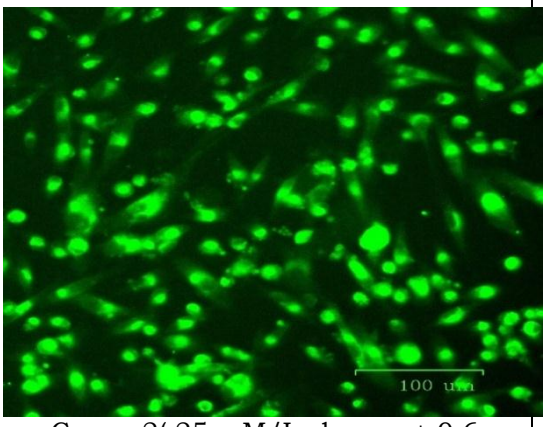


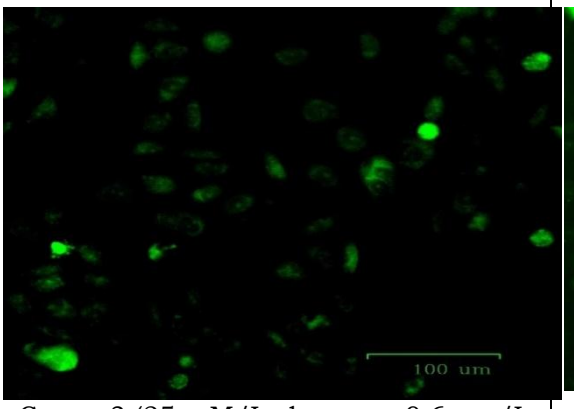
Fig 2. Oil Red O staining shows the effect of menthone on lipid accumulation in mature preadipocyte 3T3-L1 cells. The microscopic images Group1-(5 mM/L glucose + 0.6 nm/L insulin), Group2- (25 mM/L glucose + 0.6 nm/L insulin, Group3-25 mM/L glucose + 0.6 nm/L insulin + 63.22 µM/ml of Menthone), Group-4 (25 mM/L glucose + 0.6 nm/L insulin + 0.1 µM Rosiglitazone). OD at 592 of oil red staining was measured.



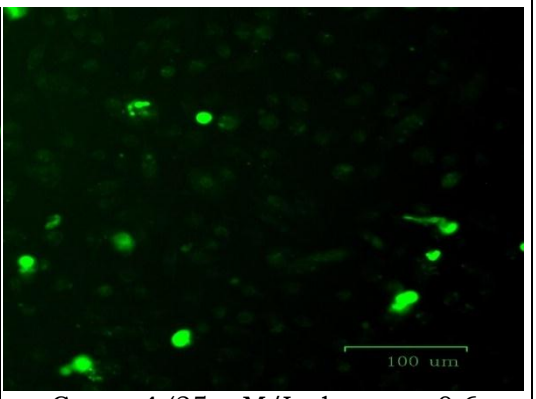
Group 1 (5 mM/L glucose + 0.6 nm/L insulin)



Group 2(25 mM/L glucose + 0.6 nm/L insulin)



Group 3 (25 mM/L glucose + 0.6 nm/L insulin + 63.22 µM/ml of Menthone)



Group 4 (25 mM/L glucose + 0.6 nm/L insulin + 0.1 µM Rosiglitazone

Fig. 3. DCFH-DA staining shows the effect of Menthone on ROS in mature preadipocyte 3T3-L1 cells.